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In re Application of: Avner YAYON et al.	Confirmation No.: 3532
Application No.: 10/761,615	Group Art Unit: 1651
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For: PLASMA PROTEIN MATRICES AND METHODS FOR THEIR PREPARATION	Attorney Docket No.: 81408-4600

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Sir:

Applicants have claimed priority under 35 U.S.C. § 119 of Israeli Patent Application No. 144446, filed July 19, 2001. In support of this claim, a certified copy of this application is submitted herewith.

No fee or certification is believed to be due for this submission. Should any fees be required, however, please charge such fees to Winston & Strawn Deposit Account No. 50-1814.

Respectfully submitted,

Date: 9-19-05

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מספר: NUMBER	144446
תאריך. Date	19 -07- 2001
הוקדם/ נדחה ANTE/POST-DATE	

בקשה לפטנט
Application For Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגד – מקום ההתאגדות)
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(בעברית) מטריצות של חלבוני פלסמה ושיטות הכנתן
(Hebrew)

(באנגלית) **PLASMA PROTEIN MATRICES AND METHODS FOR THEIR PREPARATION**
(English)

מבקש בזאת כי ינתן לי עליה פטנט
hereby apply for a patent to be granted to me in respect thereof.

*בקשה חלוקה- Division of Application	*בקשה פטנט מוסף- Addition Application for Patent	* דרישה דין קדימה Priority Claim		
מבקשת פטנט Application from	לבקשה/לפטנט to Patent/Application	מספר / סימן Number / Mark	תאריך Date	מדינת האגוד Convention Country
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המען למסירת מסמכים בישראל Address for service in Israel Webb Ben-Ami & Associates P.O.Box 2189 Rehovot 76121 תיקנו: PRO/009	ב, בן-עמי ושות' ת.ד. 2189 רחובות 76121			
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מטריצות של חלבוני פלסמה ושיטות הכנתן

PLASMA PROTEIN MATRICES AND METHODS FOR THEIR PREPARATION

PLASMA PROTEIN MATRICES AND METHODS FOR THEIR PREPARATION

FIELD OF THE INVENTION

The present invention concerns universal biomatrices comprising, inter alia, freeze dried plasma sponges and/or gels useful for clinical applications including as implants for tissue engineering as well as in biotechnology. The matrices according to the present invention may be used clinically either per se or as a cell-bearing implant.

BACKGROUND OF THE INVENTION

Guided tissue regeneration is a surgical procedure intended to restore or regenerate the morphology and function of tissues or organs that were destroyed by disease or trauma.

Matrices useful for guided tissue regeneration and/or as biocompatible surfaces useful for tissue culture are well known in the art. These matrices may therefore be considered as substrates for cell growth either in vitro or in vivo. Suitable matrices for tissue growth and/or regeneration include both biodegradable and biostable entities. Among the many candidates that may serve as useful matrices claimed to support tissue growth or regeneration, are included gels, foams, sheets, and numerous porous particulate structures of different forms and shapes.

In many instances the matrix may advantageously be composed of biopolymers, including polypeptides or proteins, as well as various polysaccharides, including proteoglycans and the like. In addition, these biopolymers may be either selected

or manipulated in ways that affect their physico-chemical properties. For example biopolymers may be cross linked either enzymatically, chemically or by other means, thereby providing greater or lesser degrees of rigidity or susceptibility to degradation.

Among the manifold natural polymers which have been disclosed to be useful for tissue engineering or culture, one can enumerate various constituents of the extracellular matrix including fibronectin, various types of collagen, and laminin, as well as keratin, fibrin and fibrinogen, hyaluronic acid, heparan sulfate, chondroitin sulfate and others. US Patents Nos. 5,955,438 and 4,971,954 disclose collagen based matrices cross linked by sugars, useful for tissue regeneration.

Damage of bone and cartilage caused by disease, such as osteoporosis and arthritis, or caused by injury, is a major cause of deformation. As further detailed below, surgical solutions for damaged cartilage include curettage, spongialization, drilling through the subchondral bone to allow pluripotent cells from the marrow space to invade and repair the injury, and transplantation of tissues such as sternal cartilage, perichondrium or periosteum. These surgical solutions showed limited success in generating neo cartilage. Recently, transplantation of isolated chondrocytes has been proposed as a specific tool for repairing articular cartilage defects in humans. However, surgery that utilizes cell transplantation methods is complicated, prolonged and can be applied only on small areas.

In the past, bone was replaced with implanted non degradable prosthetic materials, osteoconductive materials such as bone powder or ceramic materials

and steel seeded with bone cells. The success of these implants has been limited, partially due to the non degradable supports.

Fibrin, one of the plasma proteins which participates in the blood coagulation process, is known to be used in the repair of cartilage and bone defects, as follows. US Patent Nos. 6,083,383 and 5,411,885 disclose fibrin or fibrinogen glue and methods for using same. US Patent No. 4,642,120 discloses the use of fibrin or fibrinogen glue in promoting repair of defects of cartilage and bone. US Patent No. 6,074,633, on the other hand, discloses a bio-mechanical barrier for the prevention of adhesion formation that is made of sheet-like material of cross linked fibrin.

A fibrin network is formed in the blood during the coagulation process. The coagulation of blood is a complex process including the sequential interaction of a number of plasma proteins, in particular of fibrinogen, factor II, factor V, factor VII, factor VIII, factor IX, factor X, factor XI, factor XII and factor XIII. Other plasma proteins such as Von Willebrand factor (vWF), albumin, immunoglobulin, coagulation factors, and complement components may also play a part in the formation of protein net works or clots in the blood.

The clotting pathway includes a large number of individual protein species and a large number of other macromolecules in blood plasma.

Implants as a cell scaffold

Damage to cartilage may result from an inflammatory disease, such as rheumatoid arthritis, from a degenerative process or from trauma. In medicine today, the primary therapy for damaged cartilage can be divided into two main categories:

1. Techniques that stimulate the bone marrow to repair tissue, such as abrasion artheroplasty or subchondral micro-drilling that exposes the subchondral region of bone thereby allowing pluripotent bone marrow derived stem cells to initiate the healing response.

2. New techniques that emerged over the past few years that allow transplantation of new cartilage from either autologous or allogeneic sources. In more severe cases loss of cartilage can be repaired by replacement with a prosthetic material, such as silicone for cosmetic repairs, or metal alloys for joint relining. Placement of prostheses is commonly associated with significant loss of underlying tissue and bone without recovery of the full function allowed by the original cartilage, as well as the irritating presence of a foreign body. Other long-term problems associated with a permanent foreign body can include infection, erosion and instability.

Porous materials formed from synthetic and/or naturally occurring biodegradable materials have been used in the past as wound dressings or implants. The porous material provides structural support and a framework for tissue in-growth while healing progresses. Preferably, the porous material is gradually absorbed as the tissue around the wound regenerates. Typical bioabsorbable materials for use in the fabrication of porous wound dressings or implants include synthetic bioabsorbable polymers and also biopolymers such as structural proteins and polysaccharides.

The material for forming the matrix or support structure is a biodegradable artificial polymer, for example, polyglycolic acid, polyorthoester, or polyanhydride, which is degraded by hydrolysis at a controlled rate and reabsorbed. These materials provide the maximum control of degradability, manageability, size and configuration,

although other materials, including non-biodegradable materials such as Teflon can also be used. The matrix formed can be produced to assume a three dimensional configuration or a uniform sheet, and can be further overlaid with a second material such as gelatin or agarose to enhance cell attachment. The polymer matrix must be configured to provide both adequate sites for attachment and adequate diffusion of nutrients from the cell culture to maintain cell viability and growth until the matrix is implanted and vascularization has occurred.

Collagen, gelatin sponges or foams are often used as scaffolds for tissue repair in vivo and as research tools in vitro for seeding various cell types to study cell functions in three dimensions. They all have a low immunogenicity and consist of a naturally occurring structural protein to which cells can attach, interact with and degrade. In vivo they are bioabsorbable. Collagen sponges, lack biological activities typically present in the extracellular matrix environment of cells thus, is not ideal for inducing tissue regeneration.

Implants made from biological, bioabsorbable components are normally intended to be invaded by the cells of the host or recipient of the implant. Cellular invasion is required by cells which can degrade the implant materials and by those which can lay down the tissue to replace the implant and thus repair any defect which the implant is intended to repair. Failure of either type of cell to invade the structure of the implant in an efficient manner prevents vascularization which is required for new tissue to be able to sustain its life.

Liu et al. (US Patent No. 5,972,385) discloses a cross-linked collagen-polysaccharide matrix that is administered alone or in combination with other therapeutics, such as growth factors, for tissue repair. The invention also discloses that the crosslinked collagen-polysaccharide matrix is used in combination with fibrin.

The invention presents a method of preparing collagen cross-linked to an exogenous polysaccharide via aldehyde groups. The polysaccharide comprises hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan, heparan sulfate, dextran, dextran sulfate, or alginate. The preparation of the matrix include freezing and lyophilization as well as adding fibrinogen and thrombin to form fibrin in said matrix. Growth factors that are used in combination with the matrix are selected from a group consisting of members of the TGF-beta. superfamily; members of the BMP family; the growth differentiation factors(GDF's); ADMP-1; members of the fibroblast growth factor family; members of the hedgehog family of proteins; members of the insulin-like growth factor (IGF) family; members of the platelet-derived growth factor (PDGF) family; members of the interleukin (IL) family; and members of the colony-stimulating factor (CSF) family. European patent application number 98-5995/EP-A2 discloses the use of fibrin sponge containing blood clotting activator for hemostasis, cell culture support and/or wound healing.

Rosenthal et al. (US Patent No.5,700,476) relates to a bioabsorbable implant material consisting of heteromorphic (other sponge matrices are isotropic) sponge containing pharmacologically active agents, which are suitable for use as implantable materials in wound repair mainly for skin defects. The method describes the mixture of two biopolymer components, freeze dried to form a heteromorphic sponge, that will allow a phased release of the pharmacologically active agent. The biopolymer being used can be selected from a group consisting of collagen, elastin, fibronectin, vitronectin, laminin, tenascin, hyaluronic acid, chondroitin sulphate, dermatan sulphate, heparan sulphate, fibrin, oxidized regenerated cellulose, dextran and/or mixtures thereof. The active agent is selected from the group consisting of antimicrobials, cytokines, growth

factors, growth factor antagonists, antibodies, peptides, angiogenic factors, hormones, enzymes, metabolic or breakdown products of biopolymers, and pain killers.

Vacanti et al. (US Patent No. 5,736,372) discloses a biodegradable synthetic polymeric fibrous matrix containing chondrocytes for in vivo production of a functional cartilaginous structure to be used in joint lining. The cell-scaffold polymer is composed of synthetic fibers selected from the group consisting of polyanhydrides, polyorthoesters, polyglycolic acids, polylactic acids, copolymers, coated with basement membrane components such as agar, agarose, gelatin, gum arabic, collagens, fibronectin, laminin, hyaluronic acid, glycosaminoglycans, attachment peptides.

Bell et al. (US Patent No. 5,948,429) discloses a method of preparing a biopolymer foam derived from fetal pig, or alternatively can be prepared from alginic acid or polyvinyl alcohol or a protein selected from a group of collagen, laminin, fibronectin, fibrinogen, and fibrin, where the crosslinking step occurs after the freeze-drying step. A collagen solution is added to the freeze-dried foam, thereby forming a collagen-coated foam.

Naughton et al. (US Patent No. 5,842,477) discloses methods of in vivo cartilage repair by implanting a biocompatible scaffold in combination with periosteal/perichondrial tissue for two purposes; one is hold the scaffold in place, and the other to provide a source of cells that promote the induction of factors for the production of new cartilage at the site of implantation. The biodegradable scaffold used is selected from a group consisting of polyglycolic acid, polylactic acid, cellulose, methylcellulose, gelatin, hyaluronic acid, collagen or polyhydroxyalkanoates. The scaffold structure can also be composed of a non degradable material such as polyamide, a polyester, a polystyrene, a polypropylene, a

polyacrylate, a polyvinyl, a polycarbonate, a polytetrafluorethylene, polyhydroxylakanoate, cotton or a cellulose. A bioactive agent selected from a group of cellular growth factors is used to stimulate the processes involved in cartilage repair such as migration of stromal cells, chondrogenesis, stimulation of matrix deposition, suppression of inflammatory process, and immuno-suppression. The active agents consist of transforming growth factor-beta, bone morphogenetic protein that stimulates cartilage formation.

U.S. Patent No. 5,260,420, discloses a method for preparation and usage of biological glue comprising fibrin that can be injected at the site of injury. Generally it is known that fibrin has a long history as a tissue adhesive medical device, and is commercially available. Lyophilized plasma-derived protein concentrate (containing Factor XIII, fibronectin, and fibrinogen) is used in the presence of thrombin and calcium to form an injectable biological glue.

Nowhere in the background art is it taught or suggested that matrices composed of freeze dried plasma are useful for clinical applications in vivo. Furthermore, the use of freeze dried plasma matrices as a support for cellular growth and/or as an implant suitable for transplantation has never been disclosed.

SUMMARY OF THE INVENTION

The present invention provides an inexpensive, biodegradable, non immunogenic and biologically active matrix of plasma-derived proteins.

The plasma protein matrix of the invention is useful, inter alia, as a support for cellular growth and for implantation. Thus, the implantable cellular growth support and method for cell transplantation, according to the invention, do not suffer from the drawbacks of the prior art transplantation methods.

The plasma protein matrix of the invention may be a sponge or gel comprising plasma proteins. In an embodiment of the invention the plasma protein matrix is a sponge having a mean modulus of up to approximately 2,000 Kpa. In another embodiment the plasma protein matrix is a sponge having a mean modulus of up to 1,661 Kpa. The sponge matrix of the invention may have irregular pores or substantially regular pores. In the specification and the claims the term substantially regular pores means that the majority of the pores or more preferably substantially all the pores are in the same size range. More preferred matrices according to the invention have pores of a diameter in the range of 50-200 microns. Currently most preferred embodiments according to the present invention are fibrin sponge matrices with pore sizes in the range of 100 - 200 μm.

According to one embodiment of the invention, at least one of the plasma proteins used for preparing the matrix may be autologous. According to another embodiment of the invention the plasma proteins are all autologous.

The plasma matrix having the above specified mechanical properties may be obtained by any appropriate method, the currently preferred method being freeze drying a plasma protein gel.

In one embodiment of the invention the plasma matrix is prepared by mixing plasma proteins with thrombin in the presence of calcium chloride under conditions suitable for achieving gelation; applying the mixture of plasma proteins and thrombin to a solid support prior to achieving gelation; freezing the gelled mixture; and drying the gelled mixture. In one aspect of this embodiment plasma proteins are mixed with 1.5 units of thrombin per mg precipitated total plasma protein in a ratio of 3:1. The mixture is frozen at –70°C for at least a day and is then lyophilized for between 1 to 2 days.

The plasma proteins utilized in the present invention are preferably obtained from autologous plasma. However, plasma proteins from any immunologically or otherwise suitable source may be used, as well as engineered proteins or peptides having the capability to form, upon reaction with thrombin and factor XIII, a cross linked plasma protein gel. Thus, in one embodiment of the invention, the plasma proteins utilized in the present invention include at least fibrinogen and factor XIII.

Further provided by the present invention is an implant for transplanting cells in vivo. The implant consists of a plasma protein scaffold bearing cells at a density that is higher than 10^5 cells per scaffold, preferably at least 10^6 cells per scaffold. For example, an implant for transplanting chondrocytes to a site of cartilage damage consists of a 300µl, 9mm (diameter) x 2mm (thickness), plasma protein

scaffold having $1*10^6$ chondrocytes seeded therein, prior to a 2 – 3 day incubation period. Preferably, a plasma protein scaffold for transplanting chondrocytes is made of autologous plasma proteins and/or autologous chondrocytes are used for transplantation. In a particular embodiment of the invention a plasma protein scaffold for transplanting chondrocytes comprises a fibrin sponge having a substantially regular pore size of 100 – 200 μm and a mean modulus of up to approximately 1,661 KPa.

The plasma protein scaffold may also be used as an implant per se, for providing mechanical support to a defected or injured site in vivo and/or for providing a matrix for cells from the defected or injured site in vivo to expand and grow on. The plasma protein matrix of the invention, being an effective cell growing surface, may further be utilized in vivo in reconstructive surgery (for example as a matrix for regenerating neuron cells) or as a coating on synthetic or other implants such as pins and plates, for example, in hip replacement procedures. Thus, the present invention further provides a coating for implants comprising the plasma protein matrix of the invention.

Also provided by the present invention is a biocompatible surface useful for tissue culture, such as for growing chondrocytes, osteoblasts, epithelial cells, neurons an any other cell types which it is desired to culture attached to a surface.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be understood and appreciated more fully from the following detailed description taken in conjunction with the drawings in which:

Figures 1A - D show a fibrin sponge prepared according to an embodiment of the invention prior to being freeze dried (1 A), after freeze drying, prepared using low fibrinogen concentration (1B) and high fibrinogen concentration (1D) and compared to a prior art collagen sponge (1C);

Figures 2A - D show chondrocytes grown on the plasma matrix in accordance with an embodiment of the invention;

Figure 3 is a graph showing the GAG content of cells grown on a fibrin sponge according to an embodiment of the invention compared with cells grown on a fibrin gel according to an embodiment of the invention and on a prior art collagen sponge; and

Figure 4 is a graph showing the mechanical properties of a fibrin sponge according to an embodiment of the invention compared with the mechanical properties of a prior art collagen sponge.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a biodegradable matrix of plasma-derived proteins. The matrix according to an embodiment of the invention can be utilized in methods for making and/or repairing various tissues in vivo. The matrix according to a particular embodiment of the invention can be utilized in methods for making and/or repairing cartilage tissue amongst others, as a non-limitative example. Further, the matrix, according to another embodiment of the invention, can be utilized as a support for cellular growth in vivo and in implantation procedures, for example in implantation of a support carrying stromal cells, such as chondrocytes or chondrocyte progenitor cells, which cells can be seeded at the site of implantation in vivo. Further, the matrix according to other embodiments of the invention, can be utilized as an in vivo support for growth of neural cells, for example in reconstructive surgery. In yet further embodiments of the invention, the matrix can be utilized as a coating of synthetic or other implants. The matrix of the invention may be applied to implants such as pins or plates by coating or adhering methods known to persons skilled in the art. The matrix coating, which is capable of supporting and facilitating cellular growth, can thus be useful in providing a favorable environment for the implant.

The plasma protein matrix according to further embodiments of the invention can be used as a matrix for growing cells or tissue culture in vitro. As will be shown in the examples below, the matrix of the invention is a plasma protein sponge or gel. In its wet form, before drying, the matrix is a gel. In a dried form the matrix is a sponge. In both forms the matrices of the invention provide a relatively large

surface area for cells to grow on and a mechanically improved scaffold for implantation.

Thus, the matrices of the invention are useful as products for in vitro and in vivo applications.

The following examples describe an embodiment of the invention in which the plasma matrix, according to certain embodiments of the invention, is used as a support for chondrocyte growth and as a scaffold for neo cartilage formation. However, the plasma matrix of the invention may be used as a surface useful for tissue culture for any suitable cells, such as mesenchyme cells or other tissue forming cells at different levels of potency. For example, chondrocyte progenitor cells can be grown on the matrix of the invention, cells typically referred to as "stem cells" or "mesenchymal stem cell", a pluripotent, or lineage-uncommitted, progenitor cell, which is potentially capable of an unlimited number of mitotic divisions to either renew its line or to produce progeny cells which will differentiate into chondrocytes. Also a lineage-committed progenitor cell can be grown on the matrix of the invention. Such a cell is produced from the mitotic division of a stem cell and will eventually differentiate into a chondrocyte. Unlike the stem cell from which it is derived, the lineage-committed progenitor is generally considered to be incapable of an unlimited number of mitotic divisions and will eventually differentiate into a chondrocyte.

Cartilage is a specialized type of dense connective tissue comprising cells embedded in an extracellular matrix (ECM). The biochemical composition of cartilage differs according to type; however, the general composition of cartilage

comprises chondrocytes surrounded by a dense ECM consisting of collagen, proteoglycans and water. Several types of cartilage are recognized in the art, including, for example, hyaline or articular cartilage such as that found within the joints, fibrous cartilage such as that found within the meniscus and costal regions, and elastic cartilage. It will be appreciated that the matrix of the invention can support the growth and/or implantation of any type of cartilage or other suitable tissue. Furthermore, although the invention is directed predominantly to methods for growth and/or implantation of tissue in humans, the invention may also include methods for growth and/or implantation of tissues in any mammal.

The matrix of the invention, in certain embodiments may further include one or more disinfectants, such as methylene blue, and/or one or more drugs such as antibiotics.

Also anti fibrinogenic agents such as tranexamic acid, may be included in the matrix of the invention. These compounds promote fibrinolysis and thus can be used for controlling the rate of the degradation of fibrin in vivo. Further agents, such as cytokines, growth factors and their activators etc., may be included in the matrix of the invention, for example, in order to enhance a therapeutic effect.

The plasma matrix of the invention is demonstrated below as a fibrin sponge support for growing chondrocytes for implantation at a site of damaged bone or cartilage. However, the matrix may be a sponge or gel comprising other plasma proteins and may be used per se, as an implanted scaffold on to which proximal cells in vivo may perfuse and grow, or as a support for cell growth that is used for transplantation of cells to an injured tissue, or to any other suitable site in vivo.

Thus, a person skilled in the art can adjust the procedures exemplified below in accordance with specific tissue requirements.

The following examples are intended to be merely illustrative in nature and to be construed in a non-limitative fashion.

Examples

Example I. Protocol for obtaining plasma protein fractions

Protocol A

Materials:

1) Sodium citrate 3.8 %

2) Saturated ammonium sulfate ($(NH_4)_2SO_4$) (500g/l)

3) 50 mM phosphate / 10 mM EDTA, pH 6.6

3.4 g monobasic Potassium Phosphate

10 ml 0.5 M EDTA pH 7.0

Complete with DDW to 500 ml

4) 50 mM Tris / 150 mM NaCl; pH 7.4

4.35 g NaCl

25 ml Tris 1 M pH 7.5

Complete with DDW to 500 ml

5) 4°C chilled absolute Ethanol

6) Whole blood (from the Israeli blood donors' bank)

Preparation procedure:

1. One bag of blood contains 450 ml. Add 50 ml of the 3.8% sodium citrate solution to obtain 10% Vol:Vol. Mix and divide into 50 ml tubes each containing 40 ml of solution and spin at 3200 rpm in a for 20 min.

2. Collect the supernatant plasma in 50 ml tubes and re-spin it at 5000g for 15 min. at 4°C.

3. Collect the supernatant plasma in an E-beaker, put on ice, add 1 volume, of 100% saturated ammonium sulfate solution to 3 volumes of supernatant; this will yield a 25% saturated ammonium sulfate protein precipitation. 75 ml ammonium sulfate to 225 ml plasma is a typical amount. Keep the solution at 4°C for 1.5 hrs. Shake occasionally but do not stir with magnetic bar.

4. Divide 40 ml supernatant plasma into 50 ml tubes and centrifuge at 5000g for 15 min at 4°C. Remove the supernatant and wash each pellet with 10 ml 25% ammonium sulfate solution (this will not dissolve the pellet).

5. Dissolve each pellet with 6-7 ml of solution (3) (50 mM PO_4 / 10 mM EDTA pH 6.6). Keep a sample , typically 100 µl of the solution for future analysis.

6. Pool all of the solutions.

7. Repeat step 3. Use of 25 ml of 100% saturated ammonium sulfate to 75 ml plasma is a typical amount.

8. Centrifuge at 5000g for 15 min. Dissolve the pellets in solution (4) (50 mM Tris / 150 mM NaCl pH 7.4). The volume of buffer to use should be less than or equal to the volume of PO_4 buffer used in step 5 (typical amount is about 45 ml).

9. Dialyze for 3-4 hours at 4°C against 1.5 liters of solution (4). Use snakeskin dialysis tubes (Pierce). Can be done overnight.

10. Centrifuge the dialyzed sample at 14,000 rpm for 15 min to remove any insoluble material. Use high-speed resistant tubes. Keep the collected supernatant on ice.

11. Pour supernatant plasma into 50 ml tubes. Add the chilled EtOH to supernatant to a final concentration of 7%. (ex. 3.7 ml EtOH to 49 ml supernatant) (If solutions are not cold enough the precipitate will not form) and keep on ice for half an hour.

12. Centrifuge at 5000g for 15 min.

13. Dissolve pellet in the same volume added in step 8 of solution (4) (typical amount 45 ml).

14. Dialyze again overnight at 4°C against 1.5 liter of solution (4).

15. Centrifuge the solution at 14,000 rpm in the RC5 rotor for 15 min, to eliminate any non-dissolved material.

Analysis:

Determine protein concentrations by Bradford protein detection kit.

Check ability for clot formation; Add 30 µl thrombin 100 U/ml to 70 µl product 10 mg/ml- it should be clotted within 30 sec.

Demonstrate purity by running 50 µg of the purified protein sample in 5% SDS-PAGE and stained by the Coommassie blue staining procedure.

Protocol B (slight variation to Protocol A)

1) Fresh blood is spun at 3000rpm for 20 minutes;
2) Plasma is collected and centrifuged at 5000g for 15 minutes at 4°C;
3) Plasma supernatant is collected in a beaker, put on ice and one volume of 100% saturated ammonium sulfate is added to 3 volumes of the supernatant. The solution is set at 4°C for 1 – 2 hours and stirred occasionally;
4) The solution is centrifuged at 5000g for 15 minutes at 4°C. The supernatant is removed and the pellet washed with 25% ammonium sulfate and re dissolved in 20 ml of 50mM potassium phosphate/10mM EDTA pH 6.6;
5) 25% ammonium sulfate is added to the solution and the solution is set at 4°C for 1 – 2 hours and stirred occasionally;

6) The solution is centrifuged at 5000g for 15 minutes and the pellets are dissolved in 10ml of 50mM Tris/150mM NaCl pH 7.4 (T7 buffer);

7) The new solution is dialyzed overnight at 4°C and then centrifuged at 15,000rpm for 15 minutes to remove insoluble materials;

8) Chilled ethanol is added to the collected supernatant in a final concentration of 7%;

9) The mixture is centrifuged at 5000g for 15 minutes and the pellet is dissolved in T7 buffer;

10) The final solution is dialyzed again overnight at 4°C;

11) The final solution is centrifuged at 15,000 rpm to eliminate any non-dissolved material;

12) Samples of the supernatant are taken for SDS page analysis; and

13) The rest of the supernatant is collected, frozen and lyophilized over 2 days.

Example II. Preparation procedure of plasma protein scaffold

General Procedure

1) Lyophilized plasma protein is dissolved aseptically into a concentration of 20mg/ml by using 5% tranexamic acid solution;

2) Thrombin solution of 1000units/ml is diluted aseptically into 1.5 units per mg plasma protein, specifically, fibrinogen (as determined by common protein detecting methods, such as by using Bradford

reagent) (smaller or higher units can be used in these preparations) by using a 5mM calcium chloride solution;

3) The two solutions (1 and 2) are mixed together in a ration of 3:1 respectively, until the fibrinogen is cross linked to form a stable fibrin gel. The solution is poured into plates before gelation;

4) The fibrin gel is frozen at -70 °C; and

5) The frozen gel is lyophilized in aseptic conditions for 1 – 2 days.

Scaffold morphology and mechanical properties

The morphology of the plasma protein scaffold was investigated by light microscopy after sectioning the scaffold into thick specimens. Specimens were mounted on slides and were stained by eosin exclusion. The parameters of the scaffold microstructure were investigated by geometrical measurements using the light microscope. An optical micrometer measured the pore size and the distance between neighboring pores. The mechanical properties of the scaffolds were determined at 22 °C by compressing the samples at a constant deformation rate of 2mm/min, using test apparatus. The load and deformation were monitored with high accuracy down to the load of a gram and deformation of less than 0.05mm.

Cell isolation and culturing

Cartilage pieces are sectioned into small specimens, washed aseptically with PBS and then placed in a new tube containing 3ml MEM medium.

1ml containing 170 units collagenase type II is added to the cartilage pieces and the mixture is shaken gently in a 37 °C incubator over night. When most of the sample is digested the suspension is poured through sterile gauze to remove matrix debris and undigested material. The filtrate is centrifuged and washed two times to remove residual enzyme. The number of cells is determined by a hemocytometer and viability was determined by trypan blue exclusion. The cells are plated in 150 cm^2 tissue culture flasks in 30ml of culture medium at a concentration of $5*10^6$ cells/ml. Flasks are placed in a 37 °C incubator at 5% CO_2 atmosphere and 95% humidity. The culture medium is changed every two days. The cells adhere and become confluent after a week.

At confluence, the cell medium is removed and 3ml trypsin-EDTA solution are added. The solution is aspirated and placed into centrifuge tubes and centrifuged at 800g for 10 minutes. The supernatant is removed, the pellet is dispersed and the cells are counted. To create constructs, $1*10^6$ cells are seeded within the plasma protein scaffold of 9mm in diameter and a thickness of 2mm. After culturing the cells the constructs are placed in a 37 °C incubator for 1 hour and then 1ml of fresh medium is added to each. Every 2 days medium is replaced with fresh medium and every few days 3 constructs are taken to cell proliferation and differentiation analysis.

Example III Protocol for implantation of matrices-

Rabbit cartilage repair model

32 rabbits around 2.5-3 Kg each were used. The rabbits were marked with serial I.D No. 1-32, were weighed and bled 10-15 ml of blood.

One week post bleeding a cartilage defect (7mm diameter and 2mm depth, volume 75 µl) was created and biopsies from the 32 rabbits taken.

Chondrocytes are extracted from the different biopsies and are grown in culture for one month in various conditions. The flasks are marked with the same identify number of the rabbit they were extracted from.

After 4 weeks (sub-acute condition of the damaged cartilage), transplantation of the 6 experimental conditions (4 rabbits in each group) is performed and photographs of the lesions are taken before transplantation.

The rabbits are subdivided into different experimental treatment groups and are reassigned as;

A(1-4), B(1-4), D(1-4), E(1-4), F(1-4), H(1-4)

The experimental conditions are as follows:

Treatments	
Matrix A (Ortec™ Collagen) with one concentration of cells are transplanted in 4 rabbits A(1-4)	Matrix E (Ortec™ Collagen) without cells are transplanted in 4 rabbits E(1-4)
Matrix B (Plasma protein sponge) with one concentration of cells are transplanted in 4 rabbits B(1-4)	Matrix F (Plasma Protein sponge) without cells are transplanted in 4 rabbits F(1-4)

Cells with fibrin glue are transplanted in 4 rabbit, D (1-4)	Rabbits without treatment H (1-4)

Matrices of the various experimental treatments are implanted and followed up upon 6 weeks post surgery (motion, stability, effusion etc.). After 6 weeks the rabbits are sacrificed and photographs of the lesions are taken.

Sections of the repaired cartilage and normal rabbit cartilage around the lesion (after decalcification) are examined and histo-pathological examinations of the cartilage are carried out.

Reagents

1. Collagenase Type 2

Worthington biochemical corporation (Code CLS-2 Cat. No. 4147)

Stock solution:

1700 units/ml in medium (Minimum Essential Medium)

2. Culturing Cells isolated from Articular Cartilage

Reagents:

Minimal Essential Medium (MEM) Gibco BRL cat: 21090-022

Fetal Bovine Serum (FBS) Gibco BRL cat: 16000-044

L- Glutamine Solution Gibco BRL cat: 25030-024

Complete medium:

Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 2mM L-Glutamine and 100U/ml penicillin, and 100μg/ml streptomycin

Preparation of Implants of Articular Cartilage

1. Mince tissue into 3 to 4 mm pieces with a sterile scalpel
2. Wash the tissue pieces several times with Minimal Essential Medium
3. Add Collagenase (430 U/ml)
4. Incubate at 37°C for 4 to 18 hours.
5. Wash suspension 2 times by centrifugation of cells in Compete MEM medium
6. Resuspend the pellet in complete culture medium and seed the cells for propagation.
7. Seed cells (10^6 per sponge) on the matrices 3 days before implantation.

RESULTS

As can be seen in Fig.1A the fibrin gel (10 mg fibrinogen/ml, before drying) comprises small pores (in the μm size range). In the fibrin sponge (Fig. 1B) (10 mg fibrin/ml, after drying) the pores are of 100 μm size range. The difference between the pore size of a fibrin sponge of 20 mg fibrinogen/ml (Fig. 1D) and a fibrin gel of 10 mg fibrinogen/ml (Fig, 1B), is not notable and no difference was found in the cellular growth on the two scaffolds. It can be seen that the fibrin gels and sponges of the invention have a network of substantially regular pores compared with a collagen gel (Fig. 1C).

Calf chondrocytes were grown on a fibrin sponge, according to an embodiment of the invention (Fig. 2A -D). This matrix was able to support calf chondrocyte proliferation. Around 3-5 time increase in cell number was observed from an initial state (Fig. 2A) up to cell confluence (Figs. 2B through 2D). A histological section of a one month collagen implant for Hematoxylin and Eosin (not shown) showed that the cells in the surface of the collagen sponge are smaller and retain their spherical shape, compared with cells grown on a fibrin sponge (see especially Figs. 2C and 2D). In the interior space of the collagen sponge cells re-organized in tissue like structure which resembles fibro-cartilage, whereas in the fibrin sponge (Fig. 2A – D) cells are well distributed and spaced apart from each other, embedded in a thick hyaline like extracellular matrix and retain their round cell morphology typical of mature articular chondrocytes. Small islets of retained fibrin demonstrate the capacity of the cells to completely brake down the matrix and replace it with cartilagenous matrix. These results suggest that the success of tissue-like formation, in vitro, strongly depends on the specific cell-matrix material, whereas the fibrin sponge according to an embodiment of the invention is shown to be a better matrix for cartilage tissue formation than the collagen sponge.

Further, part of the cell population grown on the above matrices expressed several of the chondrocyte differentiation markers. One of several phenotypes expressed during chondrocyte differentiation is glycosamino glycan (GAG) production. Fig. 3 shows the results of the GAG content in calf chondrocytes grown on three different matrices; collagen sponges, fibrin gels (10

mg/ml) and fibrin sponges (20 mg/ml). It is apparent from the graphs presented in Fig. 3 that cells grown on the fibrin sponge and/or fibrin gel show a significantly higher GAG content than cells grown on the collagen sponge matrix.

Mechanical properties of the plasma protein matrix according to an embodiment of the invention are demonstrated in Fig. 4. The mean modulus of a fibrin gel described in the art is 210 Kpa. The dry material modulus, in accordance with an embodiment of the present invention, increases in one order of magnitude before wetting (as shown in Fig. 4).

It will be appreciated by persons skilled in the art that the present invention is not limited to what has been particularly shown and described hereinabove. Rather the scope of the present invention is defined only by the claims which follow:

CLAIMS

1. A matrix of plasma-derived proteins useful as a support for growing cells.

2. The matrix according to claim 1 wherein at least one of the plasma derived proteins is autologous.

3. The matrix according to claim 1 wherein the plasma derived proteins are autologous.

4. The matrix according to claim 1 comprising at least fibrinogen and factor XIII.

5. The matrix according to claim 4 further comprising anti fibrinogenic agents.

6. The matrix according to claim 5 comprising tranexamic acid.

7. The matrix according to claim 6 comprising approximately 5% tranexamic acid.

8. The matrix according to claim 4 further comprising substances that enhance a therapeutic effect.

9. The matrix according to claim 8 comprising substances selected from the group consisting of disinfectants, drugs, cytokines and growth factors.

10. The matrix according to claim 1 useful as a support for growing cells in vitro.

11. The matrix according to claim 10 wherein the cells are stem cells or progenitor cells.

12. The matrix according to claim 10 wherein the cells are chondrocytes.

13. The matrix according to claim 1 useful as a support for growing cells in vivo.

14. The matrix according to claim 13 wherein the cells are progenitor chondrocyte cells.

15. The matrix according to claim 1 having a mean modulus of up to approximately 2,000 Kpa.

16. The matrix according to claim 1 having a mean modulus of up to approximately 1,661 Kpa.

17. The matrix according to claim 1 having a mean modulus of approximately 1,661Kpa.

18. The matrix according to claim 1 having irregular pores.

19. The matrix according to claim 1 having substantially regular pores.

20. The matrix according to claim 1 having pores in the size range of 50-200 microns.

21. The matrix according to claim 20 wherein the pores are in the size range of 100 - 200 microns.

22. A plasma protein scaffold comprising a fibrin sponge having a mean modulus of up to approximately 2,000 Kpa.

23. The scaffold according to claim 22 wherein the mean modulus is up to approximately 1,661Kpa.

24. The scaffold according to claim 22 having substantially regular pores.

25. The scaffold according to claim 22 having irregular pores.

26. The scaffold according to claim 22 having pores in the size range of 50-200 microns.

27. The scaffold according to claim 26 wherein the pores are in the size range of 100 - 200 microns.

28. The scaffold according to claim 22 wherein the fibrin sponge comprises plasma derived proteins.

29. The scaffold according to claim 28 wherein at least one of the plasma derived proteins is autologous.

30. The scaffold according to claim 28 wherein the plasma derived proteins are autologous.

31. The scaffold according to claim 22 wherein the fibrin sponge comprises at least fibrinogen and factor XIII.

32. The scaffold according to claim 31 further comprising anti fibrinogenic agents.

33. The scaffold according to claim 32 comprising tranexamic acid.

34. The scaffold according to claim 33 comprising approximately 5% tranexamic acid.

35. The scaffold according to claim 31 further comprising substances that enhance a therapeutic effect.

36. The scaffold according to claim 35 comprising substances selected from the group consisting of disinfectants, drugs, cytokines and growth factors.

37. The scaffold according to claim 22 having cells grown thereon, useful for implanting in vivo.

38. The scaffold according to claim 37 wherein the cells are chondrocytes.

39. The scaffold according to claim 38 wherein the cells are at a density of above 10^5 cells per scaffold.

40. The scaffold according to claim 38 wherein the cells are at a density of at least 10^6 cells per scaffold.

41. A method for preparing a matrix of plasma-derived proteins useful as a support for growing cells, the method comprising the steps of:

mixing plasma proteins with thrombin in the presence of calcium chloride under conditions suitable for achieving gelation;

applying the mixture of plasma proteins and thrombin to a solid support prior to achieving gelation;

freezing the gelled mixture; and

drying the gelled mixture.

42. The method according to claim 41 wherein the plasma proteins are mixed with 1.5 units of thrombin per mg protein in a ratio of 3:1.

43. The method according to claim 41 wherein the plasma proteins comprise at least fibrinogen and factor XIII.

44. A method for preparing a scaffold useful for implantation in vivo, comprising the steps of:

mixing plasma proteins with thrombin in the presence of calcium chloride under conditions suitable for achieving gelation;

applying the mixture of plasma proteins and thrombin to a solid support prior to achieving gelation;

freezing the gelled mixture;

drying the gelled mixture, thereby obtaining a sponge;

sectioning the sponge into slices of desired diameter;

seeding the slices with cells;

growing the cells on said slices until the cells reach the density of more than 10^5 cells per slice; and

implanting the seeded slices in vivo.

45. The method according to claim 44 wherein the plasma proteins are mixed with 1.5 units of thrombin per mg protein in a ratio of 3:1.

46. The method according to claim 44 wherein the plasma proteins comprise at least fibrinogen and factor XIII.

47. The method according to claim 44 wherein the cells are chondrocytes.

48. An implant prepared according to claim 44.

49. A method for treating bone injuries, the method comprising the steps of implanting to the site of injury an implant according to claim 48.

50. A coating matrix for an implant comprising a matrix according to claim 1.

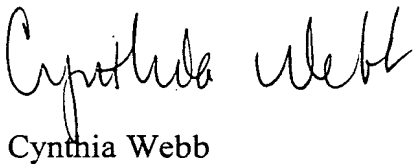
51. A coating matrix for an implant comprising a matrix according to claim 4.

52. A coating matrix for an implant comprising a matrix according to claim 5.

53. A matrix according to claim 1 comprising cross-linked fibrin.

5

For the applicants:



Cynthia Webb

Webb, Ben-Ami & Associates

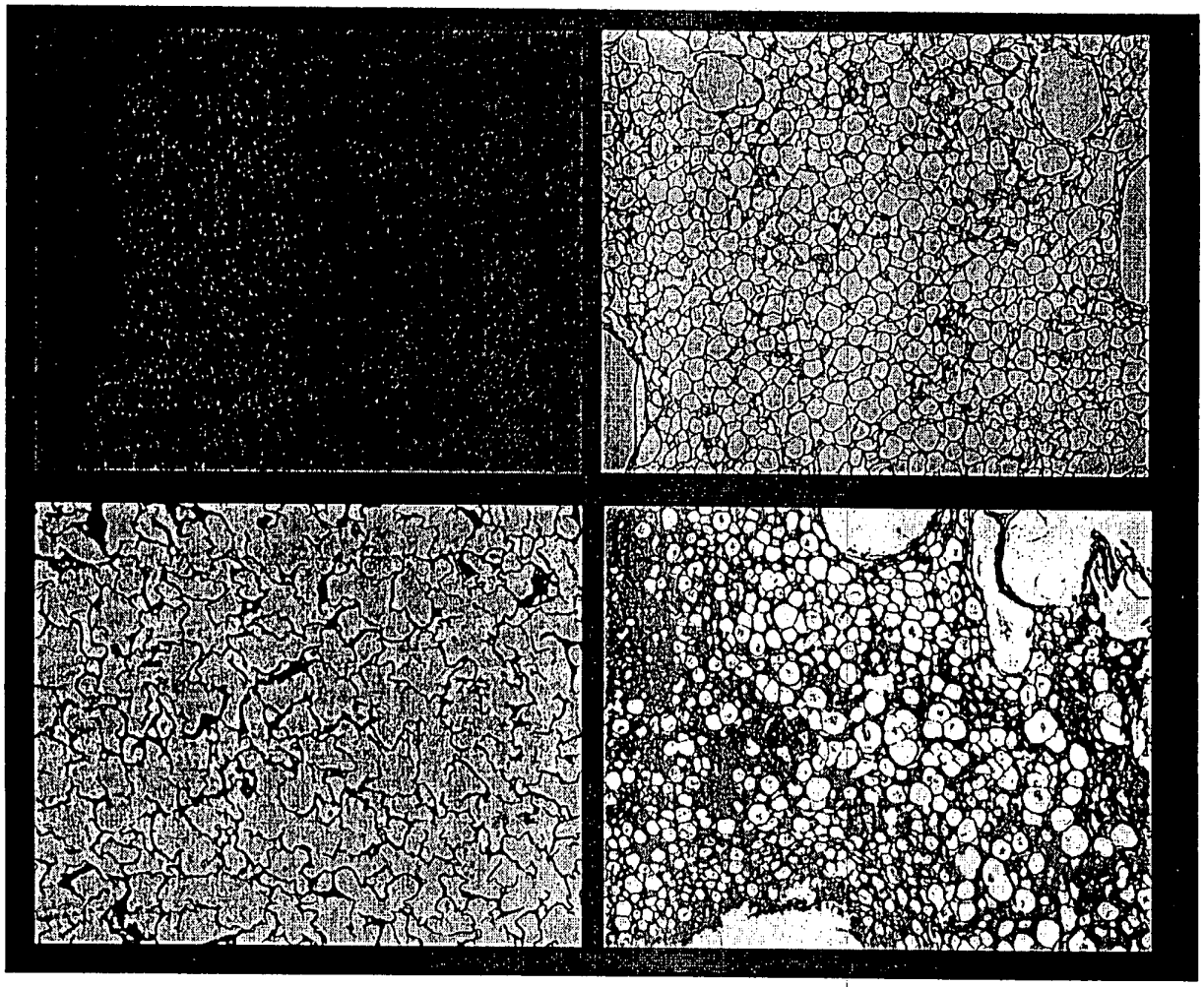


FIGURE 1

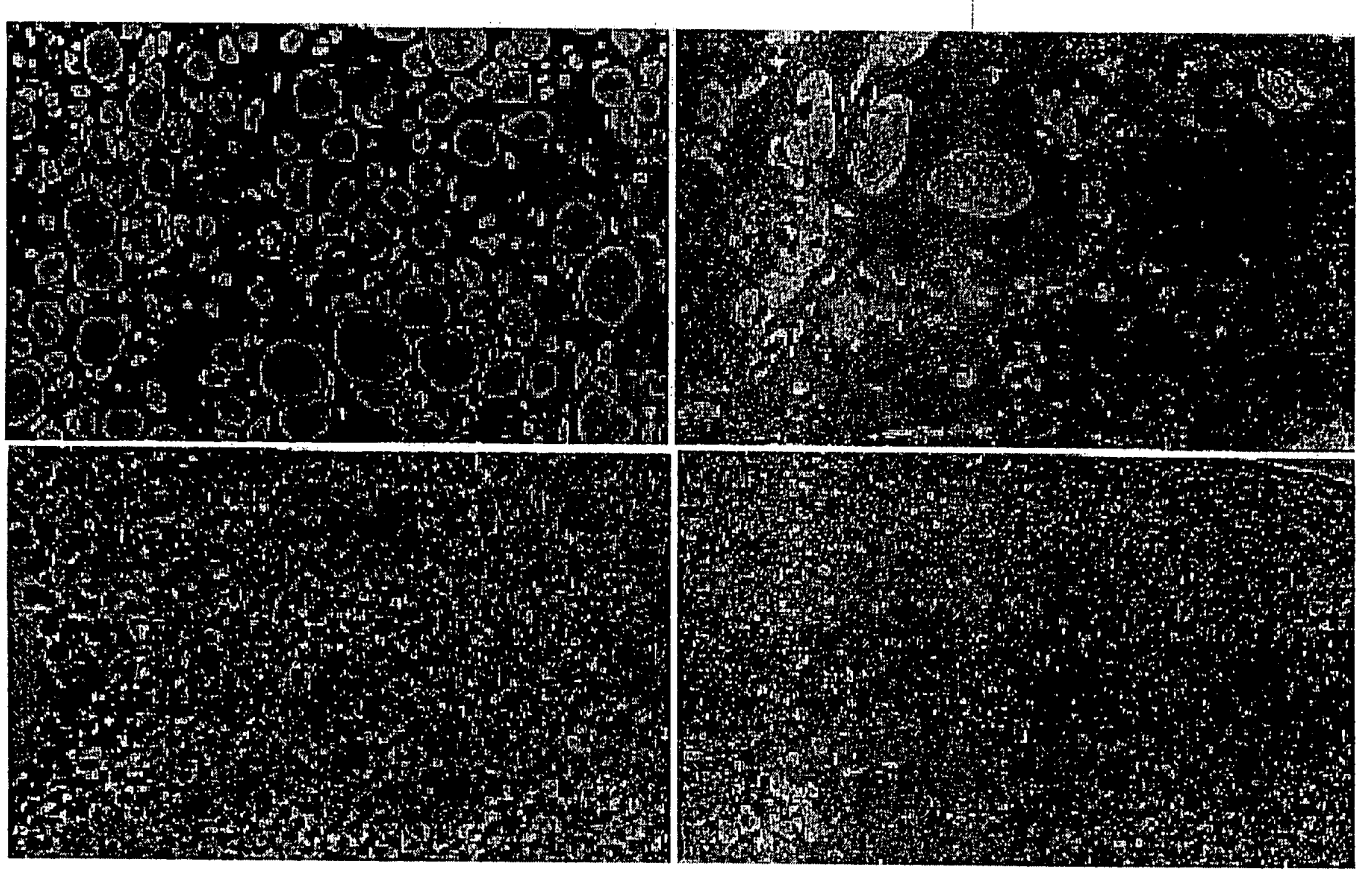


FIGURE 2

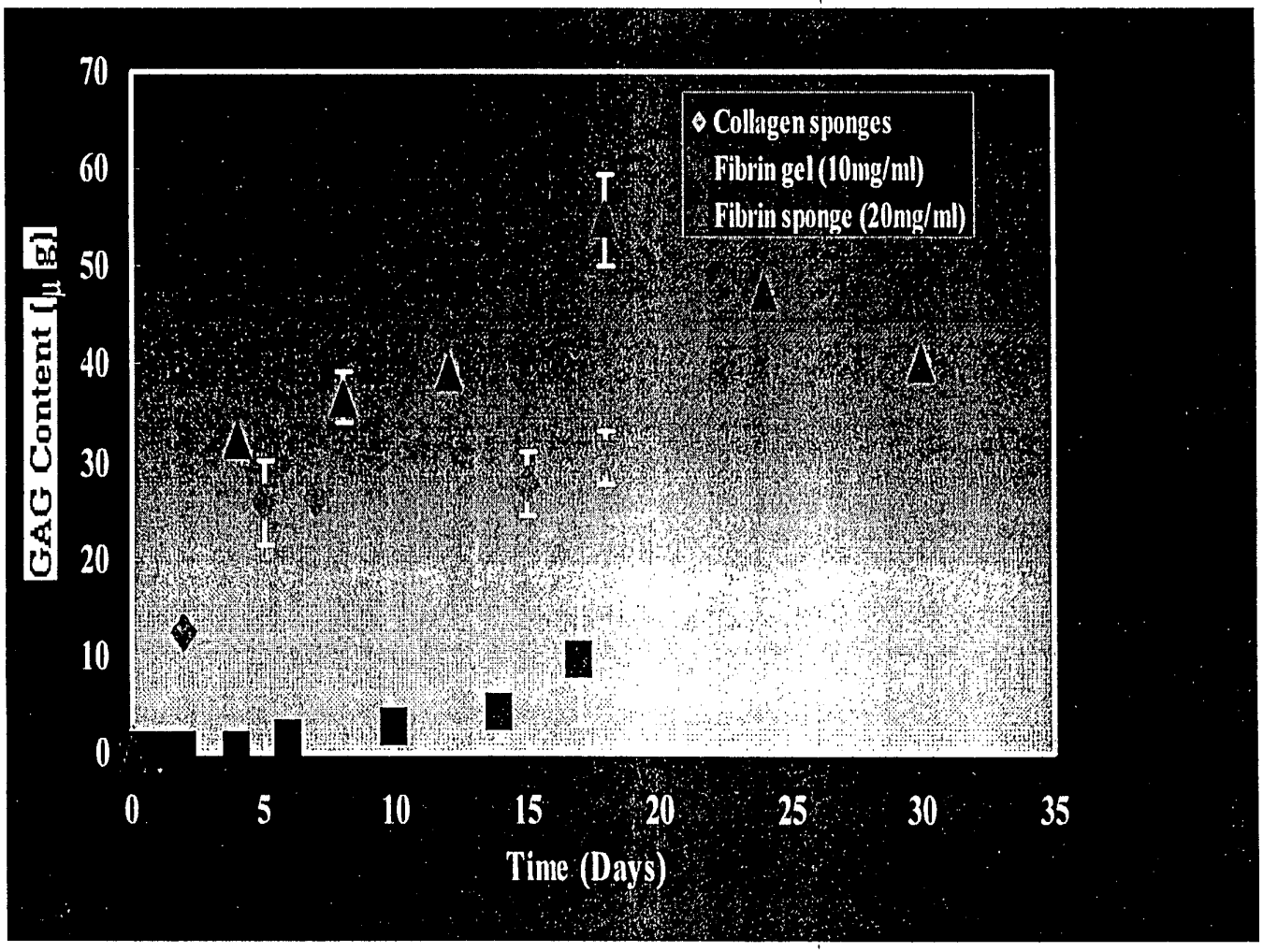

70
60
50
40
30
20
10
0
GAG Content [μg]
0
5
10
15
20
25
30
35
Time (Days)
Collagen sponges
Fibrin gel (10mg/ml)
Fibrin sponge (20mg/ml)

FIGURE 3

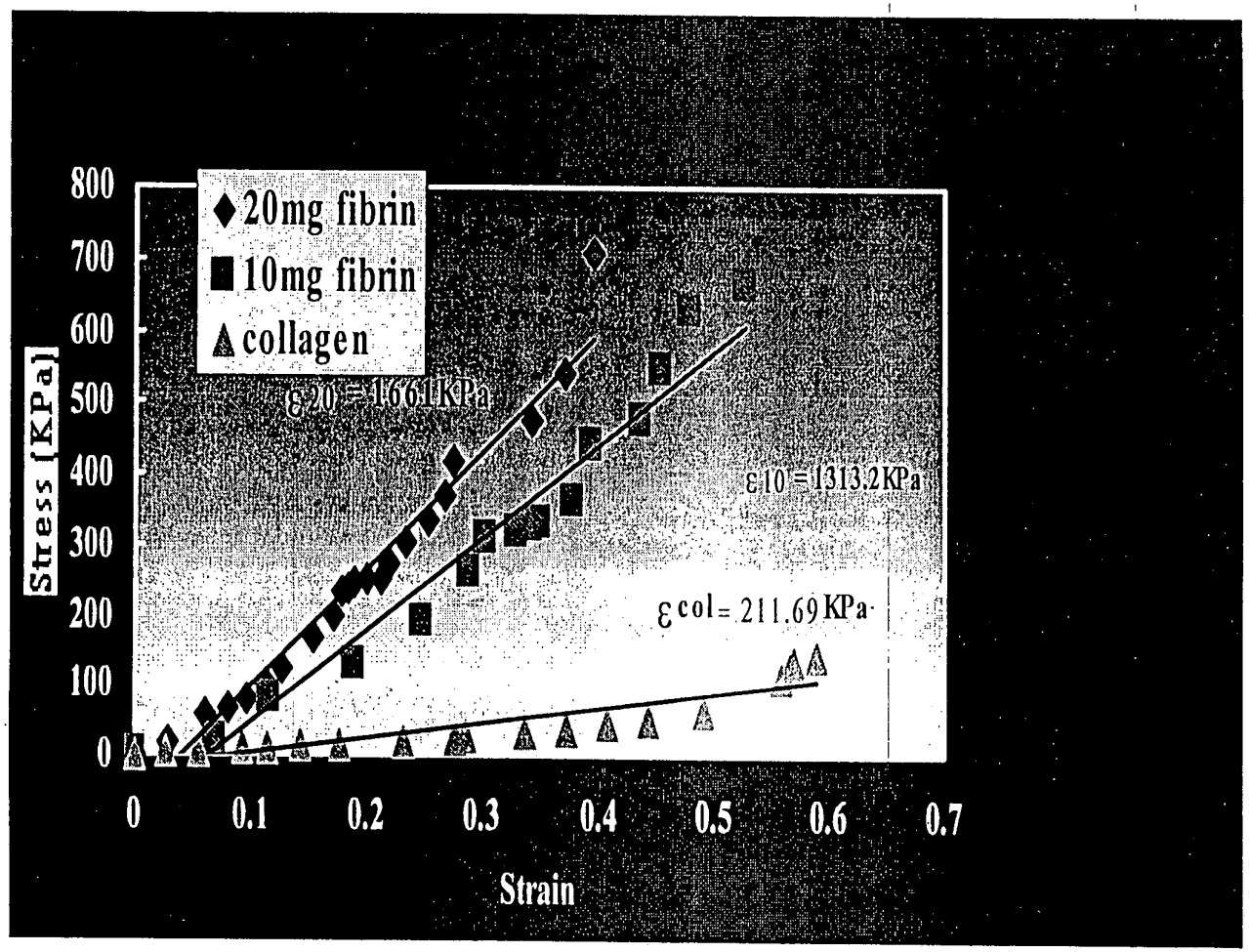

20mg fibrin
10mg fibrin
collagen
ε20 = 1661KPa
ε10 = 1313.2KPa
ε col = 211.69 KPa
Stress [KPa]
800
700
600
500
400
300
200
100
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Strain

FIGURE 4

חוק הפטנטים, תשכ"ז - 1967
1967–PATENT LAW – 5727

C07K14/75 C

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19 -07- 2001	
הוקדם/ נדחה ANTE/POST-DATE	

בקשה לפטנט
Application For Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגד – מקום ההתאגדות)
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(Hebrew)

(באנגלית) **PLASMA PROTEIN MATRICES AND METHODS FOR THEIR PREPARATION**
(English)

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hereby apply for a patent to be granted to me in respect thereof.

*בקשה חלוקה- Division of Application	*בקשה פטנט מוסף- Addition Application for Patent	* דרישה דין קדימה Priority Claim		
מבקשת פטנט Application from	לבקשה/לפטנט to Patent/Application	מספר / סימן Number / Mark	תאריך Date	מדינת האגוד Convention Country
No.....מס	No.....מס			
dated.....מיום	dated.....מיום			
* יפוי כח: מיוחד – / עד יוגש P.O.A: individual to be filed later filed in case _____ הוגש בענין				

תיקנו: PRO/009

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Signature of Applicant

For the Applicant

Cynthia Webb
Patent Attorney

היום 19 בחודש July שנת 2001
This 19 of July of the year 2001

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This form, impressed with the seal of the Patent Office and indicating the number and of filing, certifies the filing of the application the particulars of which are set out above

*מחק את המיותר - *Delete whatever is inapplicable =======